

3673-Pos**Mechanism of Regulation of Kef Channels by Chemically Diverse Glutathione Molecules**

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¹Nevada Cancer Institute, Las Vegas, NV, USA, ²University of St. Andrews, St. Andrews, United Kingdom, ³University of Aberdeen, Aberdeen, United Kingdom, ⁴University of Oxford, Oxford, United Kingdom. KTN (RCK) domains are ubiquitous, canonically dimeric, cytoplasmic regulatory domains that control the flux of K⁺ transporters and channels in response to cellular cues. We have determined the structures of the C-terminal KTN-bearing domain of KefC, bound alternately to the inhibitory ligand, reduced glutathione, or an activating glutathione adduct. Analysis of these structures reveals that the former stabilizes an inter-domain association between helix $\alpha 2$ from one KTN domain with helices $\alpha 7/8$ of the partnering chain. In contrast, activating glutathione conjugates disrupt this interaction. The resulting conformational change directly impacts the physical exposure of the KTN protein interaction interface that is critical to controlling ion flow through the pore. The elucidated mechanism explains both how subtle chemical differences in glutathione derivatives can have contrary effects on transporter function, as well as how chemically diverse adducts can all elicit activation of this system.

Cyclic Nucleotide-gated Channels**3674-Pos****Ligand Binding and Activation Gating in CNGA2A4B1b Channels**

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The olfactory cyclic nucleotide-gated (CNG) channels are heterotetramers composed of three homologue subunits, CNGA2, CNGA4, CNGB1b. The CNGA4 and CNGB1b subunits produce functional channels only in heterotetramers with CNGA2. The channels are activated by cyclic nucleotides as cAMP or cGMP. So far the contribution of the different subunits to channel activation has not been elucidated. It is even not known whether the ligands bind to the CNGA2 subunits only or also to the CNGA4 and CNGB1b subunit, because both have a cyclic nucleotide binding domain in their C-terminus.

CNGA2A4B1b channels were expressed in *Xenopus* oocytes and they were studied in excised patches by monitoring ligand binding and gating under both steady-state and non-steady state conditions. Ligand binding was measured by confocal patch-clamp fluorimetry using a fluorescent cGMP analogue (fcGMP). Similar to the homotetrameric CNGA2 channels, we observed also in heterotetrameric channels a crossover of the normalized steady-state binding and steady-state activation. Moreover, the binding curve of the CNGA2A4B1b channels closely approximates the binding curve of the homotetrameric channels.

In order to study the binding to the β subunits we used two approaches: first we expressed either CNGA4 or CNGB1b alone in oocytes and secondly we coexpressed either CNGA4 or CNGB1b with a mutated CNGA2 subunit that has decreased sensitivity to cGMP. Using patch-clamp fluorimetry with either fcGMP or fcAMP no significant binding was observed to the CNGA4 and CNGB1b subunits. Furthermore, activation time courses following [cGMP] or [cAMP] jumps were considered. The cGMP-induced activation of CNGA2A4B1b channels was up to 5 times slower than that by cAMP over a wide concentration range whereas in CNGA2 channels they were similar. Our results seem to suggest that CNGA2A4B1b channels are activated by ligand binding to the two CNGA2 subunits only. Other possibilities are discussed.

3675-Pos**Site-Specific Protein Labeling using Reversible Transition Metal Ion Binding**

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Fluorescence spectroscopy is a powerful tool for studying the structure and conformational dynamics of protein molecules both in isolation and in their cellular context. Fluorescence experiments frequently employ small, cysteine-re-

active fluorophores. However, it can be difficult to obtain specific labeling of a desired cysteine in proteins with multiple cysteines or in a protein's native environment in which many cysteine-containing proteins are present. To obtain specific labeling, we have developed a method where a desired cysteine can be reversibly protected by binding transition metal ions (e.g. Cd²⁺ and Zn²⁺) while background cysteines are blocked with non-fluorescent covalent modifiers. Following removal of transition metal ions, the deprotected cysteine is then available to specifically react with a fluorophore. In order to protect specific cysteines, the affinity for metal binding was increased by placing metal binding residues nearby in regions of known secondary structure (e.g. an α -helix) to act as coordination partners in a metal binding site. The placement of histidine residues next to or one turn away from a cysteine in an α -helix increased the metal binding affinity of that cysteine. This allows for protection of a particular cysteine at much lower metal concentrations. Using pairs of cysteines, rather than a combination of cysteines and histidines, further stabilized metal binding. Finally, we demonstrate the ability of this technique to selectively label a particular cysteine in mixtures of proteins containing cysteines. These motifs are simple, can be introduced into proteins with minimal perturbation, and allow for selective labeling of sites within a protein, specific proteins in a mixture, or proteins in a native environment. This technique has great potential for use in patch-clamp fluorimetry studies of ion channels in which non-specific labeling of patch-associated proteins makes it difficult to resolve fluorescence from channel proteins.

3676-Pos**Cyclic Nucleotide Gated Channels have a Functional Voltage Sensor Disabled by Hydrophobic Interactions**

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Voltage-gated Na⁺, K⁺, Ca²⁺ and cyclic nucleotide-gated (CNG) channels belong to the same super-family of ion channels. All these channels harbour a voltage sensor but their gating is different: the opening of CNG channels is poorly dependent on membrane voltage, whereas the gating of Na⁺, K⁺, Ca²⁺ channels is highly voltage-dependent. By using electrical recordings in voltage clamp mode, we show that in wild type CNGA1 channels the voltage sensor does not move as in K⁺ channels. However, when the hydrophobic interaction coupling Phe380 in S6 to Leu356 in the P-helix is impaired, the voltage sensor moves as in K⁺ channels and the gating of mutant channels F380A and L356A becomes voltage dependent. Voltage gating in mutant channels F380A is accompanied by nonlinear capacitive transients at the beginning and termination of voltage stimuli, reminiscent of gating currents measured in Kv channels, suggesting dipole rearrangement or charge translocation caused by the applied electrical field. Moreover, when Arg272 and Arg275 in the S4 segment are neutralized one at time in a F380A background, significant changes of the characteristic activation parameters can be observed, in agreement with what described for Kv and Nav channels. Therefore, we can conclude that the voltage sensor in CNG channels is blocked by the tight coupling between the P-helix and S6, necessary condition that makes the channel gated by binding of cyclic nucleotides and not by changes of membrane voltage.

3677-Pos**Interaction Energies between Intracellular Regions in CNG Channel Activation**

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Cyclic nucleotide-gated (CNG) channels mediate sensory signal transduction in retinal and olfactory cells. The channels are activated by the binding of cyclic nucleotides to an intracellular cyclic nucleotide-binding domain (CNBD). The molecular events translating the binding to the pore opening are still unknown. We investigated the role of intracellular channel regions on the activation process by constructing chimeric channels in which the N-terminus, the S4-S5 linker, the C-terminus, and the CNBD of the retinal CNGA1 subunit were systematically replaced by respective regions of the olfactory CNGA2 subunit. Macroscopic concentration-response relations were analyzed, yielding the apparent affinity to cGMP and the Hill coefficient. The degree of functional coupling of intracellular regions in the activation gating was determined by an interaction energy according to the principles of thermodynamic double-mutant cycle analysis. We show that all four intracellular regions, including the short S4-S5 linker, are involved in controlling the apparent affinity of the channel to cGMP and, moreover, in determining the degree of cooperativity between the subunits as determined from the Hill coefficients. The interaction energies are specific for pairs of regions. The interaction energy of the S4-S5 region with